RESEARCH ARTICLE

MycoKeys
A peer-reviewed open-access journal
Launched to accelerate biodiversity research

Morphological and molecular phylogenetic analyses reveal three species of *Colletotrichum* in Shandong province, China

Taichang Mu[1], Zhaoxue Zhang[1], Rongyu Liu[1],
Shubin Liu[1], Zhuang Li[1], Xiuguo Zhang[1], Jiwen Xia[1]

I *Shandong Provincial Key Laboratory for Biology of Vegetable Diseases and Insect Pests, College of Plant Protection, Shandong Agricultural University, Taian, 271018, China*

Corresponding author: Jiwen Xia (zhenjunxue@126.com)

Citation: Mu T, Zhang Z, Liu R, Liu S, Li Z, Zhang X, Xia J (2021) Morphological and molecular phylogenetic analyses reveal three species of *Colletotrichum* in Shandong province, China. MycoKeys 85: 57–71. https://doi.org/10.3897/mycokeys.85.75944

Abstract

Colletotrichum has numerous host range and distribution. Its species are important plant pathogens, endophytes and saprobes. *Colletotrichum* can cause regular or irregular depressions and necrotic lesions in the epidermal tissues of plants. During this research *Colletotrichum* specimens were collected from Mengyin County, Shandong Province, China. A multi-locus phylogenetic analysis of ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS sequence data combined with morphology, revealed a new species and two known species, viz. *C. mengyinense* sp. nov., *C. gloeosporioides* and *C. pandanicola*, belonging to the *C. gloeosporioides* species complex. The new species is described and illustrated in this paper and compared with taxa in the *C. gloeosporioides* species complex.

Keywords

Colletotrichum, Glomerellaceae, multi-gene phylogeny, new species, taxonomy

Introduction

Colletotrichum species (Glomerellaceae, Glomerellales) is one of the ten economically most important fungal plant pathogens worldwide (Dean et al. 2012). It was first observed by Tode (1790), who divided it into *Vermicularia*. Corda (1831) established *Colletotrichum* based on the characteristic of the conidiomata with setae in *Vermicularia*.

Colletotrichum is based on the type species *Colletotrichum lineola* which was associated with a member of the *Apiaceae* (Jayawardena et al. 2017). The sexual morph belongs to *Glomerella*. The asexual morph is characterized by acervuli born in the skin of the host, often producing brown sharp setae, colorless or brown conidiophores with separate, conidia colorless, pseudomonas, cylindrical or crescent-shaped (Damm et al. 2009).

Currently, more than 900 epithets of *Colletotrichum* are listed in Index Fungorum (http://www.indexfungorum.org/; accessed 22 November 2021). *Colletotrichum* has been studied for more than 200 years and the classification of *Colletotrichum* has undergone major changes (Jayawardena et al. 2016). In order to clarify its complex nature, the species are classified into 14 species complexes (Bhunjun et al. 2021). Specifically, *C. gloeosporioides* has been considered as a complex species for a long time.

The name *C. gloeosporioides* was first proposed by Penzig based on *Vermicularia gloeosporioides* which was collected from *Citrus* in Italy (Weir et al. 2012). Early in the study of *C. gloeosporioides* species complex, taxonomic concepts used were based on apparent features such as morphological characters, host species, size and shape of conidia and appressoria, presence or absence of setae, aspect, color and growth rate in culture, whether or not the teleomorph develops, etc (Weir et al. 2012). Nonetheless, Sutton commented that "no progress in the systematics and identification of isolates belonging to this complex is likely to be made based on morphology alone". Fortunately, with the development of molecular systematics, gene method is applied to taxonomy of *Colletotrichum* complexes. Multi-gene phylogeny analysis is of great significance to the study of the classification of *C. gloeosporioides* species complex and related concepts of species (Cannon et al. 2012; Damm et al. 2012; Weir et al. 2012).

The aim of this study was to explore the diversity of *Colletotrichum* species from symptomatic leaves and diseased fruit of plants in Shandong Province, China. We present a new species and two known species, *C. mengyinense* sp. nov., *C. gloeosporioides* and *C. pandanicola* based on phylogenetic data and morphology.

Materials and methods

Isolation and morphological studies

The samples were collected from Mengyin County, Shandong Province, China. The strains of *Colletotrichum* were isolated from symptomatic leaves of *Rosa chinensis* and diseased fruit of *Juglans regia* using single spore and tissue isolation methods (Chomnunti et al. 2014). The spore suspension was obtained and spread onto PDA plate and incubated for one day under the biochemical incubator. After germination, the spores were transferred to a new PDA plate to obtain pure culture. Additionally, the surface sterilized plant tissue isolation was used to obtain sterile isolates from the host plant. About 25 mm² tissue fragments were taken from the margin of tissue lesions and

surface sterilized by consecutively immersing in 75% ethanol solution for 60 s, 5% sodium hypochlorite solution for 30 s, and then rinsed in sterile distilled water for 60 s (Gao et al. 2013; Liu et al. 2015). The surface sterilized plant tissue was dried with sterilized paper and moved on the PDA plate (Cai et al. 2009). All the PDA plates were incubated at biochemical incubator at 25 °C for 3–4 days, then hyphae were picked out of the periphery of the colonies and inoculated on to new PDA plates.

Following 5–14 days of incubation, morphological characters were recorded (Cai et al. 2009). Photographs of the colonies were taken at 7 days and 14 days using a digital camera (Canon G7X). Micromorphological characters of colonies were observed using stereomicroscope (Olympus SZX10) and microscope (Olympus BX53), both fitted with high definition color digital cameras to photo document conidia and so on of fungal structures. All *Colletotrichum* strains were stored in 10% sterilized glycerin and sterile water at 4 °C for deep studies in the future. Every specimen was deposited in the Herbarium of the Department of Plant Pathology, Shandong Agricultural University (HSAUP). Living cultures were deposited in the Shandong Agricultural University Culture Collection (SAUCC). Taxonomic information of the new taxa was submitted to MycoBank (http://www.mycobank.org).

DNA extraction and amplification

Genomic DNA was extracted from *Colletotrichum* fungal mycelia grown on PDA after 5–7 days, using a modified cetyltrimethylammonium bromide (CTAB) buffer, and then it was incubated at 65 °C for 30 min with occasional gentle inverting (Guo et al. 2000). Gene sequences were obtained from seven genes loci including the internal transcribed spacer regions with intervening 5.8S nrRNA gene (ITS), partial glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), partial chitin synthase 1 gene (CHS-1), partial actin gene (ACT), partial beta-tubulin gene (TUB2), partial calmodulin gene (CAL) and partial glutamine synthetase gene (GS) were amplified and sequenced using primers pairs (Table 1).

PCR was performed using an Eppendorf Master Thermocycler (Hamburg, Germany). Amplification reactions were performed in a 25 μL reaction volume which contained 12.5 μL 2× Taq Plus Master Mix II (Vazyme, Nanjing, China), 1 μL of each forward and reverse primer (10 μM) (Tsingke, Qingdao, China), and 1 μL template genomic DNA in amplifier, and were adjusted with distilled deionized water to a total volume of 25 μL. PCR parameters were as follows: 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at a suitable temperature for 30 s, extension at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The annealing temperature for each gene was 52 °C for ITS and GS, 59 °C for CAL, 60 °C for GAPDH, 58 °C for ACT and CHS-1, 55 °C for TUB2. The PCR products were visualized on 1% agarose electrophoresis gel. Sequencing was conducted by the Tsingke Company Limited (Qingdao, China) bi-directionally. Consensus sequences were obtained using MEGA 7.0 (Kumar et al. 2016). All sequences generated in this study were deposited in GenBank (Table 2).

Table 1. Gene regions and respective primer pairs used in the study.

Locus	Gene	Primer	Direction	Sequence (5'-3')
The internal transcribed spacer regions with intervening 5.8S nrRNA gene	ITS	ITS5	Forward	GGA AGT AAA AGT CGT AAC AAG G
		ITS4	Reverse	TCC TCC GCT TAT TGA TAT GC
Partial glyceraldehyde-3-phosphate dehydrogenase gene	GAPDH	GDF1	Forward	GCC GTC AAC GAC CCC TTC ATT GA
		GDR1	Reverse	GGG TGG AGT CGT ACT TGA GCA TGT
Partial chitin synthase 1 gene	CHS-1	CHS-79F	Forward	TGG GGC AAG GAT GCT TGG AAG AAG
		CHS-354R	Reverse	TGG AAG AAC CAT CTG TGA GAG TTG
Partial actin gene	ACT	ACT-512F	Forward	ATG TGC AAG GCC GGT TTC GC
		ACT-783R	Reverse	TAC GAG TCC TTC TGG CCC AT
Partial beta-tubulin gene	TUB2	Bt-2a	Forward	GGT AAC CAA ATC GGT GCT GCT TTC
		Bt-2b	Reverse	ACC CTC AGT GTA GTG ACC CTT GGC
Partial calmodulin gene	CAL	CL1	Forward	GAR TWC AAG GAG GCC TTC TC
		CL2A	Reverse	TTT TTG CAT CAT GAG TTG GAC
		CL1C	Forward	GAA TTC AAG GAG GCC TTC TC
		CL2C	Reverse	CTT CTG CAT CAT GAG CTG GAC
Partial glutamine synthetase gene	GS	GSLF3	Forward	GAT ACG CCT CTT CCA GCG TT
		GSLR1	Reverse	AGR CGC ACA TTG TCA GTA TCG

Phylogenetic analyses

Novel sequences were generated from the nine strains in this study, and all reference available sequences of *Colletotrichum* species were downloaded from GenBank. Multiple sequence alignments for ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS were constructed and carried out using the MAFFT v.7.11 online programme (http://mafft.cbrc.jp/alignment/server/, Katoh et al. 2019) with the default settings, and manually corrected where necessary. To establish the identity of the isolates at species level, phylogenetic analyses were conducted individually for each locus and then as combined analyses of seven loci (ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS). Phylogenetic analyses were based on maximum likelihood (ML) and Bayesian.

Inference (BI) for the multi-locus analyses. For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analyses. ML and BI were run on the CIPRES Science Gateway portal (https://www.phylo.org/) using RaxML-HPC2 on XSEDE (8.2.12) (Miller et al. 2012; Stamatakis 2014) and MrBayes on XSEDE (3.2.7a), respectively (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012). For ML analyses the default parameters were used and BI was carried out using the rapid bootstrapping algorithm with the automatic halt option. Bayesian analyses included seven parallel runs of 5,000,000 generations, with the stop rule option and a sampling frequency of 1000 generations. The burn-in fraction was set to 0.25 and posterior probabilities (PP) were determined from the remaining trees. The resulting trees were plotted using FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and edited with Adobe Illustrator CS6.0. New sequences generated in this study were deposited at GenBank (https://www.ncbi.nlm.nih.gov; Table 2).

Table 2. Species and GenBank accession numbers of DNA sequences used in this study with new sequences in bold.

Species	Strain/Isolate	Host/Substrate	GenBank accession number						
			ITS	GAPDH	CHS-1	ACT	TUB2	CAL	GS
Colletotrichum aenigma	ICMP 18608*	*Persea americana*	JX010244	JX010044	JX009774	JX009443	JX010389	JX009683	JX010078
C. aeschynomenes	ICMP 17673*=ATCC 201874	*Aeschynomene virginica*	JX010176	JX009930	JX009799	JX009483	JX010392	JX009721	JX010081
C. alatae	CBS 304.67*=ICMP 17919	*Dioscorea alata*	JX010190	JX009990	JX009837	JX009471	JX010383	JX009738	JX010065
C. alienum	ICMP 12071*	*Malus domestica*	JX010251	JX010028	JX009882	JX009572	JX010411	JX009654	JX010101
C. aotearoa	ICMP 18735	*Hedychium gardnerianum*	JX010221	JX010023	JX009880	JX009500	JX010424	JX009620	JX010015
C. areciola	hb8	*Areca catechu*	MW561344	MW557464	-	-	MW557482	-	-
C. artocarpicola	MFLUCC18-1167*	*Artocarpus heterophyllus*	MN415991	MN435568	MN435569	MN435570	MN435567	-	-
C. asianum	ICMP 18580*=CBS 130418	*Coffea arabica*	FJ972612	JX010053	JX009867	JX009584	JX010406	FJ917506	JX010096
C. australianum	BRIP 63695	*Capsicum annuum*	KU923677	MN442115	MW092000	MN442105	KU923693	-	KU923737
C. boninense (outgroup)	CBS 123755*	*Crinum asiaticum* var. *sinicum*	JQ005153	JQ005240	JQ005327	JQ005501	JQ005588	-	-
C. camelliae	ICMP 10643	*Camellia × williamsi*	JX010224	JX009908	JX009891	JX009540	JX010436	JX009630	JX010119
C. changpingense	MFLUCC 15-0022*	*Fragaria × ananassa*	KP683152	KP852469	KP852449	KP683093	KP852490	-	-
C. chiangmaiense	MFLUCC 18-0945	*Magnolia garrettii*	MW346499	MW548592	MW623653	MW655578	-	-	-
C. chrysophilum	CMM4268*	*Musa* sp.	KX094252	KX094183	KX094083	KX093982	KX094285	KX094063	KX094204
C. ciggaro	ICMP 19122	*Vaccinium* sp.	JX010228	JX009950	JX009902	JX009536	JX010433	JX009744	JX010134
C. clidemiae	ICMP 18658*	*Clidemia hirta*	JX010265	JX009989	JX009877	JX009537	JX010438	JX009645	JX010129
C. cobbittiense	BRIP66219	*Cordyline stricta × Cordyline australis*	MH087016	MH094133	MH094135	MH094134	MH094137	-	-
C. conoides	CAUG17*	*Capsicum annuum*	KP890168	KP890162	KP890156	KP890144	KP890174	-	-
C. cordylinicola	MFLUCC090551*=ICMP 18579	*Cordyline fruticosa*	JX010226	JX009975	JX009864	HM470235	JX010440	HM470238	JX010122
C. dracaenigenum	MFLUCC 19-0430*	*Dracaena fragrans*	MN921250	MT215577	MT215575	MT313686	-	-	-
C. endophytica	CAUG28	*Capsicum annuum*	KP145441	KP145413	KP145385	KP145329	KP145469	-	-
C. fici-septica	MFLU 19-27708*	*Ficus septica*	MW114367	MW183774	MW177701	MW151585	-	-	-
C. fructicola	MFLU 090228*	*Coffea arabica*	FJ972603	FJ972578	-	FJ907426	FJ907441	FJ917508	FJ972593
C. fructivorum	CBS 133125*	*Vaccinium macrocarpon*	JX145145	-	-	-	JX145196	-	-
C. gloeosporioides	IMI356878*=ICMP 17821	*Citrus sinensis*	JX010152	JX010056	JX009818	JX009531	JX010445	JX009731	JX010085
	ICMP 19121	*Citrus limon*	JX010148	JX010054	JX009903	JX009558	-	JX009745	-
	SAUCC200952	***Juglans regia***	**MW786743**	**MW876474**	**MW883689**	**MW883698**	**MW888973**	**MW922541**	**MW888964**
	SAUCC200954	***Juglans regia***	**MW786744**	**MW876475**	**MW883690**	**MW883699**	**MW888974**	**MW922542**	**MW888965**
	SAUCC201001	***Juglans regia***	**MW786745**	**MW876477**	**MW883692**	**MW883701**	**MW888976**	**MW922544**	**MW888967**
C. grevilleae	CBS 132879*	*Grevillea* sp.	KC297078	KC297010	KC296987	KC296941	KC297102	KC296963	-
C. grossum	CAUG7*	*Capsicum* sp.	KP890165	KP890159	KP890153	KP890141	KP890171	KP890147	-
C. bebeiense	MFLUCC130-726*	*Vitis vinifera*	KF156863	KF377495	KF289008	KF377532	KF288975	-	-
C. hedericola	MFLU 15-0689	*Hedera helix*	MN631384	-	MN635794	MN635795	-	-	-
C. belleniense	CBS 142418*	*Poncirus trifoliata*	KY856446	KY856270	KY856186	KY856019	KY856528	-	-
C. henanense	LF238*	*Camellia sinensis*	KJ955109	KJ954810	-	KM023257	KJ955257	KJ954662	KJ954960

Species	Strain/Isolate	Host/Substrate	GenBank accession number						
			ITS	GAPDH	CHS-1	ACT	TUB2	CAL	GS
C. horii	ICMP 10492	Diospyros kaki	GQ329690	GQ329681	JX009752	JX009438	JX010450	JX009604	JX010137
C. hystricis	CPC 28153*	Citrus hystrix	KY856450	KY856274	KY856190	KY856023	KY856532	-	-
C. jiangxiense	LF687*	Camellia sinensis	KJ955201	KJ954902	-	KJ954471	KJ955348	KJ954752	KJ955051
C. kahawae	IMI 319418*=ICMP 17816	Coffea arabica	JX010231	JX010012	JX009813	JX009452	JX010444	-	JX010130
C. ledongense	CGMCC3.18888*	Quercus palustris	MG242008	MG242016	MG242018	MG242014	MG242010	-	-
C. makassarense	CBS 143664a*=CPC 28612	Capsicum annuum	MH728812	MH728820	MH805850	MH781480	MH846563	-	-
C. mengyinense	**SAUCC200702***	**Rosa chinensis**	**MW786742**	**MW846240**	**MW883686**	**MW883695**	**MW888970**	**MW922538**	**MW888961**
	SAUCC200912	**Juglans regia**	**MW786689**	**MW876472**	**MW883687**	**MW883696**	**MW888971**	**MW922539**	**MW888962**
	SAUCC200913	**Juglans regia**	**MW786690**	**MW876473**	**MW883688**	**MW883697**	**MW888972**	**MW922540**	**MW888963**
	SAUCC200983	**Juglans regia**	**MW786642**	**MW876476**	**MW883691**	**MW883700**	**MW888975**	**MW922543**	**MW888966**
C. musae	CBS 116870*=ICMP 19119	Musa sp.	JX010146	JX010050	JX009896	JX009433	HQ596280	JX009742	JX010103
C. nupharicola	CBS 470.96*=ICMP 18187	Nuphar lutea subsp. polysepala	JX010187	JX009972	JX009835	JX009437	JX010398	JX009663	JX010088
C. pandanicola	MFLU 18-0003*	Pandanus sp.	MG646967	MG646934	MG646931	MG646938	MG646926	-	-
	SAUCC200204*	**Juglans regia**	**MW786641**	**MW846239**	**MW883685**	**MW883694**	**MW888969**	**MW922537**	**MW888960**
	SAUCC201152	**Juglans regia**	**MW786746**	**MW876478**	**MW883693**	**MW883702**	**MW888977**	**MW922545**	**MW888968**
C. perseae	GA100*	Persea americana	KX620308	KX620242	-	KX620145	KX620341	KX620206	KX620275
C. proteae	CBS 132882*	Protea sp.	KC297079	KC297009	KC296986	KC296940	KC297101	KC296960	-
C. pseudotheobromicola	MFLUCC 18-1602	Prunus avium	MH817395	MH853675	MH853678	MH853681	MH853684	-	-
C. psidii	ICMP 19120	Psidium sp.	JX010219	JX009967	JX009901	JX009515	JX010443	JX009743	JX010133
C. queenslandicum	ICMP 1778*	Carica papaya	JX010276	JX009934	JX009899	JX009447	JX010414	JX009691	JX010104
C. rhexiae	CBS 133134*	Rhexia virginica	JX145128		-		JX145179	-	-
C. salsolae	ICMP 19051*	Salsola tragus	JX010242	JX009916	JX009863	JX009562	JX010403	-	JX010094
C. siamense	ICMP 18578*	Coffea arabica	JX010171	JX009924	JX009865	FJ907423	JX010404	FJ917505	JX010094
	ICMP 19118	Jasminum sambac	HM131511	HM131497	JX009895	HM131507	JX010415	-	JX010105
C. syzygicola	MFLUCC10-0624*	Syzygium samarangense	KF242094	KF242156	-	KF157801	KF254880	KF254859	-
C. tainanense	CBS 143666*	Capsicum annuum	MH728818	MH728823	MH805845	MH781475	MH846558	-	-
C. temperatum	Coll883*	Vaccinium macrocarpon	JX145159				JX145211		
C. theobromicola	ICMP 18649	Theobroma cacao	JX010294	JX010006	JX009869	JX009444	JX010447	JX009591	JX010139
C. ti	ICMP 4832*	Cordyline sp.	JX010269	JX009952	JX009898	JX009520	JX010442	JX009649	JX010123
C. tropicale	CBS 124949*=ICMP 18653	Theobroma cacao	JX010264	JX010007	JX009870	JX009489	JX010407	JX009719	JX010097
C. viniferum	GZAAS5.08601*	Vitis vinifera	JN412804	JN412798	-	JN412795	JN412813	-	-
C. wuxiense	CGMCC 3.17894*	Camellia sinensis	KU251591	KU252045	KU251939	KU251672	KU252200	-	KU252101
C. xanthorrhoeae	BRIP 45094*=ICMP 17903 = CBS 127831	Xanthorrhoea preissii	JX010261	JX009927	JX009823	JX009478	JX010448	JX009653	JX010138
C. yulongense	CFCC 50818*	Vaccinium dunalianum	MH751507	MK108986	MH793605	MH777394	MK108987	MH793604	MK108988
Colletotrichum sp.	BRIP 58074a	Citrus australasica	MK469999	MK470017	MW091975	MK470089	MK470053	-	MK470035

Strains marked with "*" are ex-type or ex-epitype.

Results

Phylogenetic analyses

Nine strains of *Colletotrichum* isolated from leaves of *Rosa chinensis* and fruit of *Juglans regia* in Mengyin County, Shandong Province, China, were grown in culture. Among the nine *Colletotrichum* isolates were identified a new species and two known species based on an analysis of combined ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS gene sequences composed of 69 isolates of *C. gloeosporioides* species complex and *C. boninense* (CBS 123755) as the outgroup taxon.

A total of 3953 characters including gaps were obtained in the phylogenetic analysis, viz. ITS: 1–619, GAPDH: 620–929, CHS-1: 930–1229, ACT: 1230–1542, TUB2: 1543–2288, CAL: 2289–3028, GS: 3029–3953. Of these characters, 2667 were constant, 674 were variable and parsimony-uninformative, and 612 were parsimony-informative.

The Bayesian analysis lasted 4,685,000 generations, resulting in 4686 total trees, of which 3515 trees were used to calculate the posterior probabilities. The BI posterior probabilities were plotted on the ML tree. For the BI and ML analyses, HKY+G for GAPDH and ACT, SYM+I+G for ITS, K80+I+G for CHS-1, GTR+G for GS and CAL, HKY+I for TUB2 were selected and incorporated into the analyses. The ML tree topology confirmed the tree topologies obtained from the BI analyses, and therefore, the ML tree is presented (Fig. 1).

ML bootstrap support values (≥ 50%) and Bayesian posterior probability (≥ 0.90) are shown as first and second position above nodes, respectively. The 70 strains were assigned to 60 species clades based on the seven gene loci phylogeny (Fig. 1). The nine strains studied here represented a novel species and two known species. The new species of *C. mengyinense* showed a close relationship to *C. fructicola* (MFLU 090228) with full support (ML-BS: 100% and BYPP: 1). The strains SAUCC200952, SAUCC200954 and SAUCC201001 belong to *C. gloeosporioides* (IMI356878) with full support (ML-BS: 100% and BYPP: 1) by the multi-locus phylogeny. The strains SAUCC200204 and SAUCC201152 belong to *C. pandanicola* (MFLU 18-0003) with good support (ML-BS: 94% and BYPP: 0.99) by the multi-locus phylogeny.
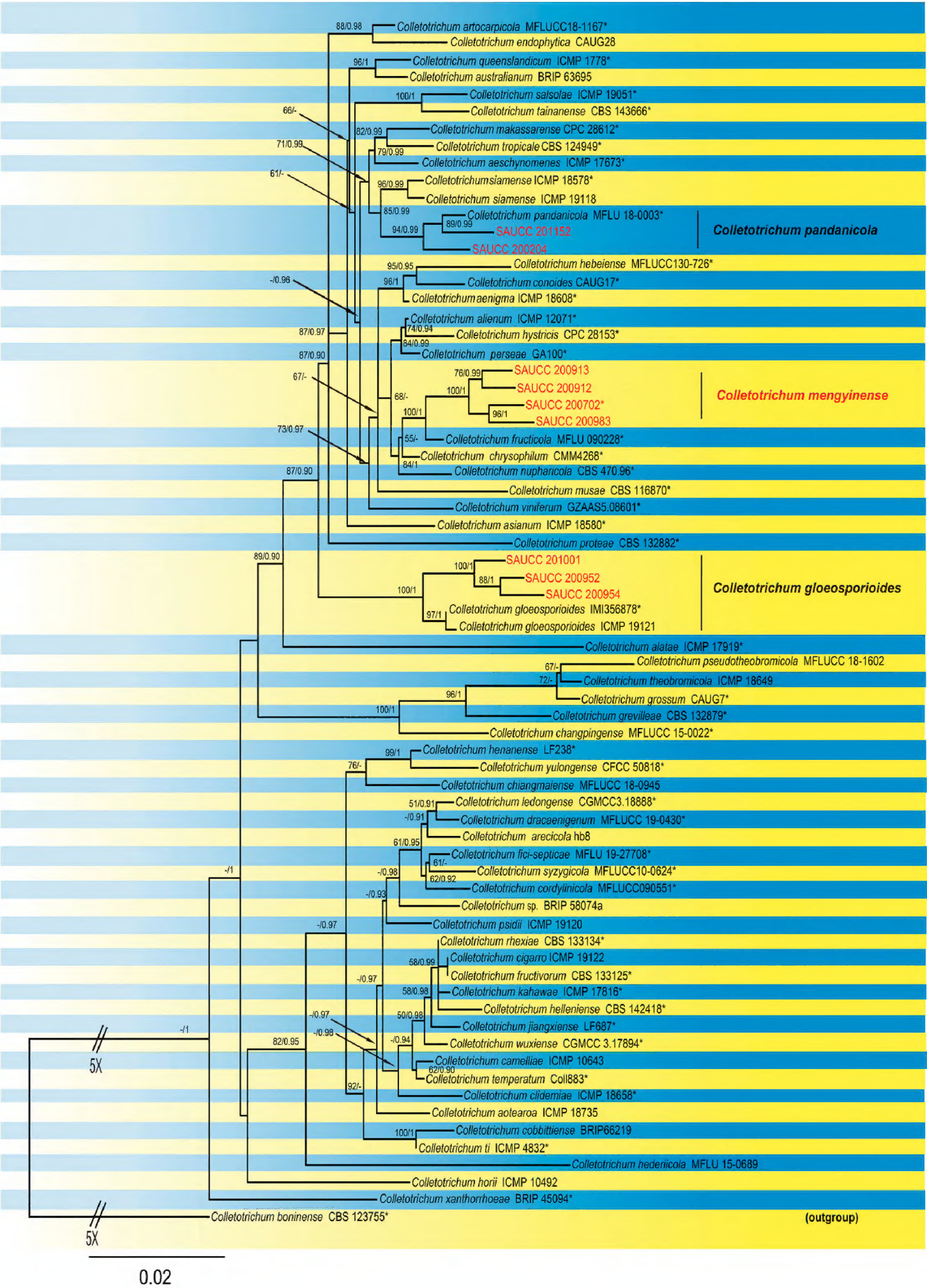
Taxonomy

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., Atti Reale Ist. Veneto Sci. Lett. Arti., ser. 6, 2: 670. 1884
Figure 2

*Vermicudaria gloeosporioides* Penz., Michelia 2: 450, 1882. Basionym.

Description. Lesion fruit, round or irregular, dark brown slightly sunken center, brown at margin. Asexual morph developed on PDA. A mass of orange conidia grows in the white my-

Figure 1. Phylogram of *Colletotrichum gloeosporioides* complex based on combined ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS genes. The ML and BI bootstrap support values above 50% and 0.90 BYPP are shown at the first and second position, respectively. Strains marked with "*" are ex-type or ex-epitype. Strains from this study are shown in red. Two branches were shortened to fit the page size-these are indicated by the symbol (//) with an indication number showing how many times they are shortened.
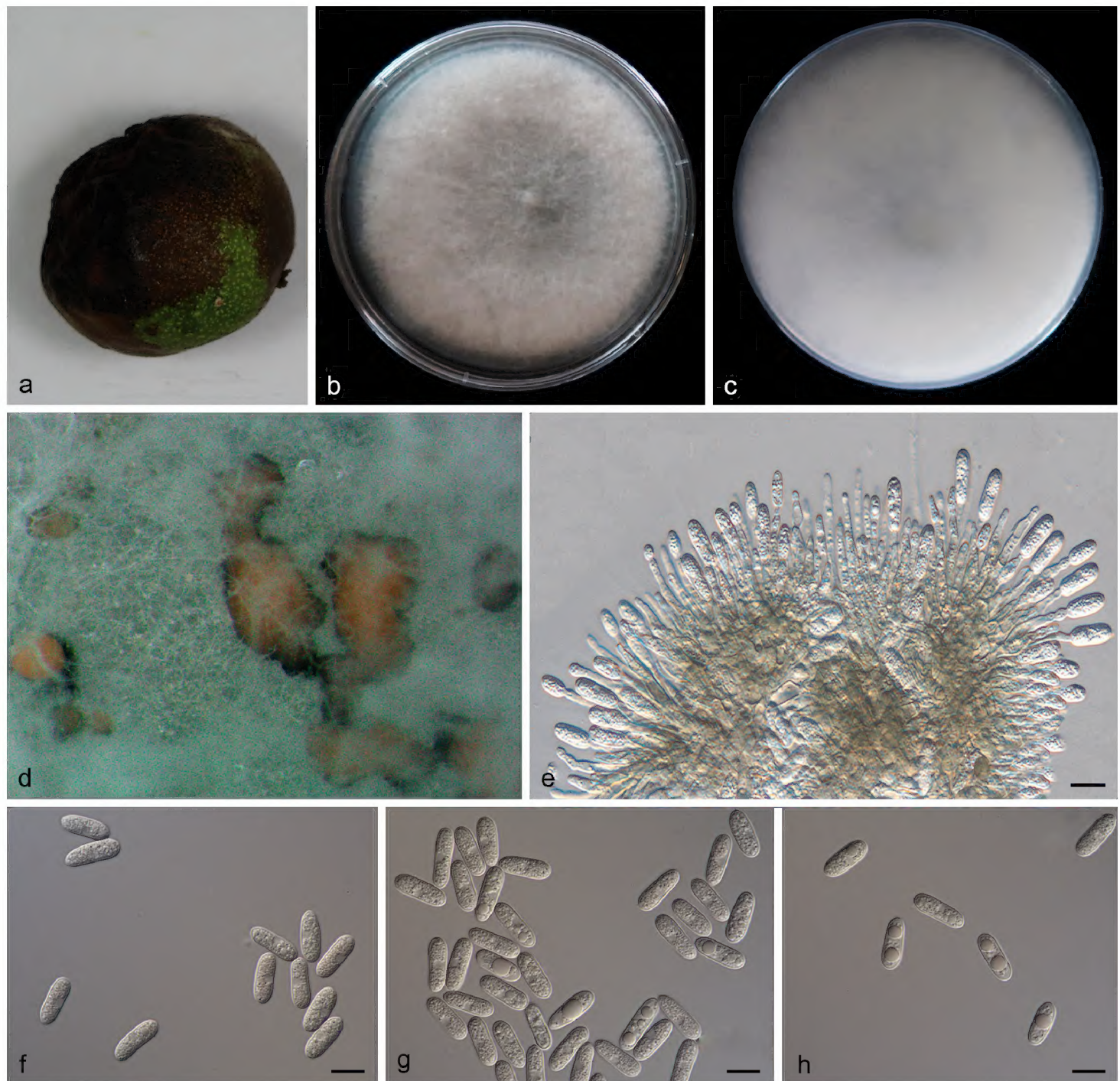
Figure 2. *Colletotrichum gloeosporioides* (SAUCC201001) **a** lesion fruit of host plant **b, c** surface (**b**) and reverse (**c**) sides of colony after incubation for 7 days on PDA **d** conidiomata **e** conidiophores, conidiogenous cells and conidia **f–h** conidia. Scale bars: 10 μm (**e–h**).

celium of PDA after 14 days in light at 25 °C. Conidia, hyaline, smooth-walled, subcylindrical, both ends round, 1–3-guttulate, contents granular. Conidia on PDA (10.6–16.5 × 4.3–5.3 μm, mean ± SD = 14.9 ± 1.5 × 4.9 ± 0.3 μm, L/W ratio = 3.0, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, straight to curved, 4.7–12.7 × 3.1–4.0 μm, opening 1.5–2.0 μm diam. Conidiophores hyaline, smooth walled, septate, branched.

Culture characteristics. Colonies on PDA flat with entire margin, aerial mycelium white, floccose cottony; surface and reverse grayish in the center and white margin. PDA attaining max 81 mm in diameter after 7 days, at 25 °C, growth rate 8.7–11.5 mm/day. Colonies on SNA sparse hyphae, slow growth.

Specimens examined. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200952, ex-paratype living culture SAUCC200952. China, Shandong Province: Mengyin

County, Mengshan, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200954, ex-paratype living culture SAUCC200954. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia* 25 July 2020, T.C. Mu, paratype HSAUP201001, ex-paratype living culture SAUCC201001.

Notes. *Colletotrichum gloeosporioides* was originally described as *Vermicularia gloeosporioides* on fruit of *Citrus sinensis* in Italy and this species placed in *Colletotrichum* by Corda (Weir et al. 2012; Cannon et al. 2008). In the present study, three strains (SAUCC200952, SAUCC200954 and SAUCC201001) are clustered to *C. gloeosporioides* clade in the combined phylogenetic tree (Fig. 1). Morphologically, our strains were similar to *C. gloeosporioides* by conidia (10.6–16.5 × 4.3–5.3 vs. 12.0–17.0 (–23.5) × 4.5–6.0 μm, mean：14.9 × 4.9 vs. 14.4 × 5.6 μm). We therefore consider the isolated strain as *C. gloeosporioides*.

Colletotrichum mengyinense T.C. Mu, J.W. Xia, X.G. Zhang & Z. Li, sp. nov.

MycoBank No: 841265
Figure 3

Etymology. Named after Mengyin County where the fungus was collected.

Diagnosis. *Colletotrichum mengyinense* can be distinguished from the phylogenetically most closely related species *C. fructicola* (MFLU 090228) by its large conidia (12.5–15.7 × 4.8–6.1 vs. 9.7–14.0 × 3.0–4.3 μm), and five loci (2/509 in the ITS region, 1/139 GAPDH, 9/237 ACT, 8/410 TUB2 and 20/727 GS).

Type. China, Shandong Province: Mengyin County, on diseased leaves of *Rosa chinensis*, 25 July 2020, T.C. Mu, holotype HSAUP200702, ex-type living culture SAUCC200702.

Description. Leaf spots discoid to irregular, brown or tanned. Asexual morph developed on SNA. A yellowish or orange mass appearing just as accumulations of conidia on the surface of the medium of SNA after 14 days in light at 25 °C. Conidia one-celled, hyaline, smooth-walled, subcylindrical, both ends round, contents granular. Conidia on SNA (12.5–15.7 × 4.8–6.1 μm, mean ± SD = 14.3 ± 1.1 × 5.3 ± 0.4 μm, L/W ratio = 2.7, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, hyaline, 5.3–15.5 × 2.9–4.9 μm, opening 1.7–2.5 μm diam. Conidiophores hyaline, smooth walled, septate, branched.

Culture characteristics. Colonies on PDA flat with entire margin, aerial mycelium white or gray, floccose cottony; surface and reverse gray in the center and grayish margin. PDA attaining 69.3–75.6 mm in diameter after 7 days, at 25 °C, growth rate 9.9–10.8 mm/day. Colonies on SNA sparse hyphae, slow growth.

Additional specimen examined. China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200912, ex-paratype living culture SAUCC200912. China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200913, ex-paratype living culture SAUCC200913. China, Shandong Province: Mengyin County, on diseased fruit of *Juglans regia*, 25 July 2020, T.C. Mu, paratype HSAUP200983, ex-paratype living culture SAUCC200983.
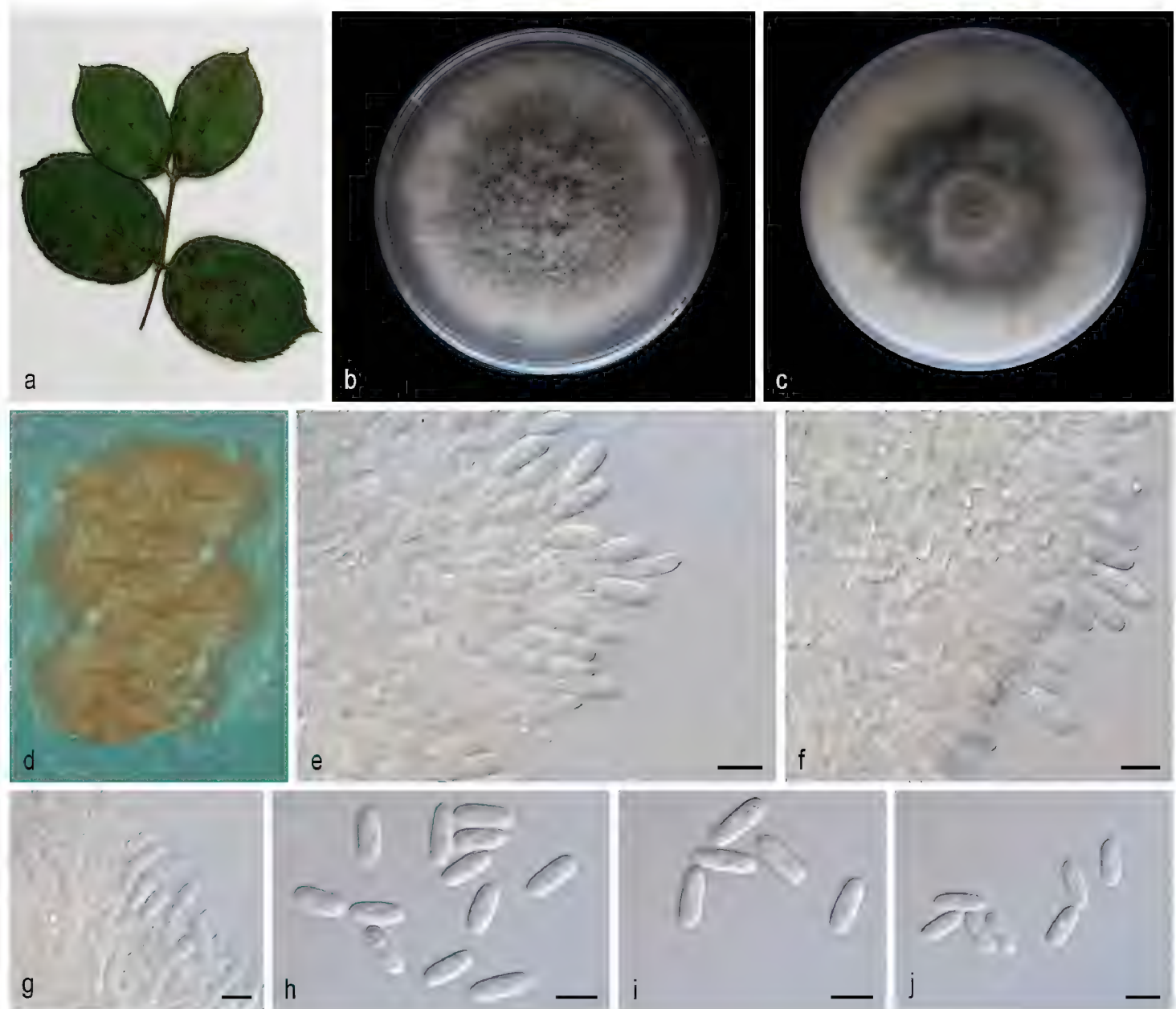
Figure 3. *Colletotrichum mengyinense* (SAUCC200702) **a** branch with leaves of host plant **b, c** surface (**b**) and reverse (**c**) sides of colony after incubation for 7 days on PDA **d** conidiomata **e-g** conidiophores, conidiogenous cells and conidia **h–j** conidia. Scale bars: 10 μm (**e–j**).

Notes. Phylogenetic analysis of a combined seven gene showed that *Colletotrichum mengyinense* formed an independent clade (Fig. 1) and is phylogenetically distinct from *C. fructicola* (Prihastuti et al. 2009). This species can be distinguished from *C. fructicola* by 40 different nucleotides (2/509 in the ITS region, 1/139 in the GAPDH region, 9/237 ACT, 8/410 TUB2 and 20/727 GS). What's more, *C. mengyinense* differs from *C. fructicola* in having large conidia (12.5–15.7 × 4.8–6.1 vs. 9.7–14.0 × 3.0–4.3 μm, mean: 14.3 × 5.3 vs. 11.53× 3.55 μm). Therefore, we establish this fungus as a novel species.

Colletotrichum pandanicola Tibpromma & K.D. Hyde, MycoKeys 33:47. (2018)
Figure 4

Description. Lesion fruit, round or irregular, dark brown slightly sunken center, brown at margin. Asexual morph developed on SNA. A mass of yellowish or orange
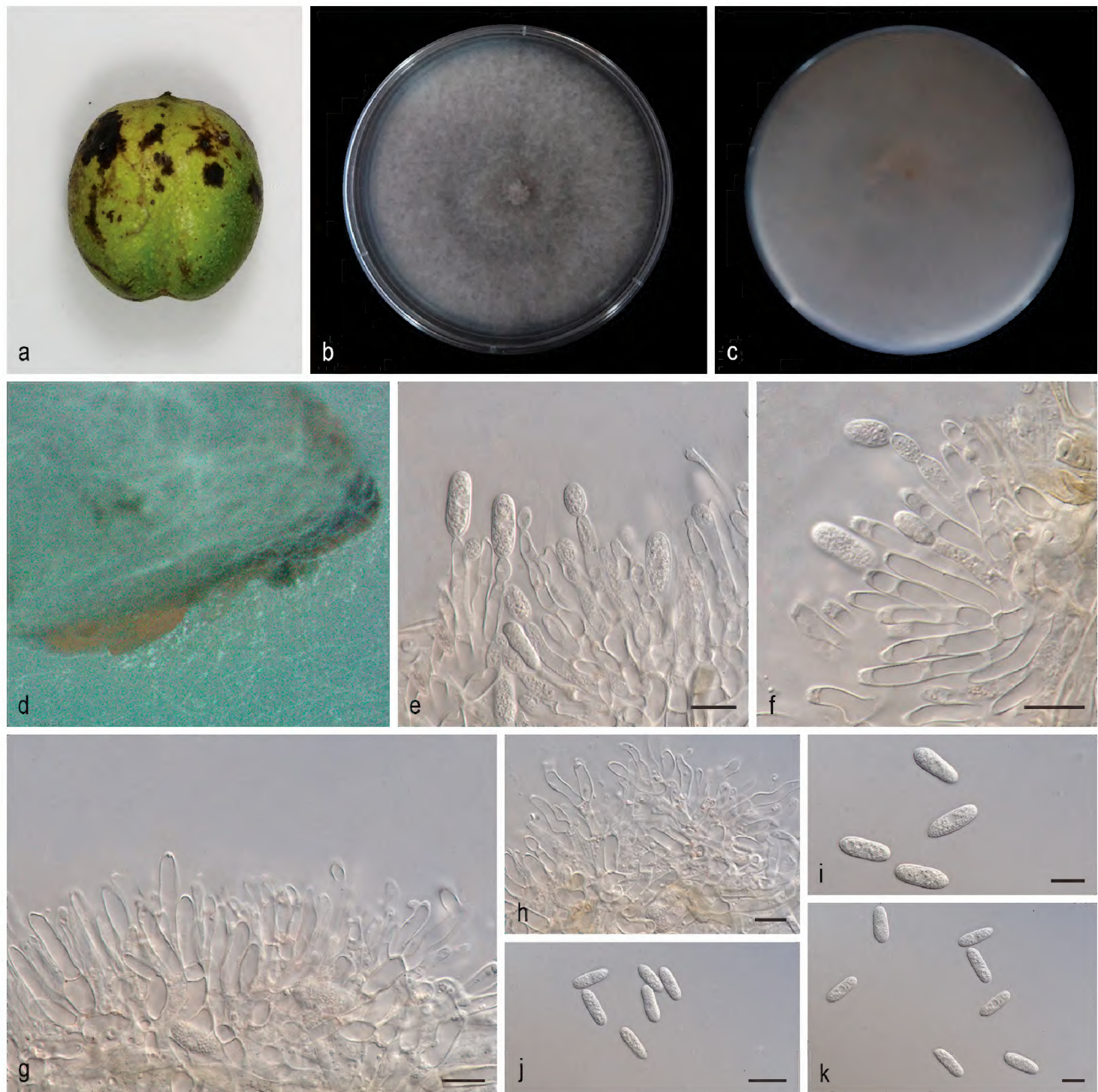
Figure 4. *Colletotrichum pandanicola* (SAUCC201152) **a** lesion fruit of host plant **b, c** surface (**b**) and reverse (**c**) sides of colony after incubation for 7 days on PDA **d** conidiomata **e, f** conidiophores, conidiogenous cells and conidia **g, h** conidiophores, conidiogenous cells **i–k** conidia. Scale bars: 10 μm (**e–k**).

creamy conidial droplets at the inoculum point on SNA after 14 days in light at 25 °C. Born in conidiomata, conidia first take an ovoid shape, then become subcylindrical with rounded ends, contents granular. Conidia on SNA (14.2–17.9 × 4.6–6.0 μm, mean ± SD = 16.1 ± 0.9 × 5.4 ± 0.3 μm, L/W ratio = 2.9, n = 40). Sexual morph not observed. Conidiogenous cells subcylindrical, hyaline, 5.5–23.9 × 2.6–6.3 μm, opening 1.1–1.5 μm diam. Conidiophores branched, hyaline, smooth walled, septate, some septa disappeared at the end, contents granular.

Culture characteristics. Colonies on PDA flat with entire margin, aerial mycelium white, floccose cottony; light gray in the center and pale white margin, reverse white to pale brownish. PDA attaining 58.1–82.6 mm in diameter after 7 days, at 25 °C, growth rate 8.3–11.8 mm/day. Colonies on SNA sparse hyphae, slow growth.

Specimens examined. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia*. 25 July 2020, T.C. Mu, paratype HSAUP200204, ex-paratype living culture SAUCC200204. China, Shandong Province: Mengyin County, Mengshan, on diseased fruit of *Juglans regia*. 25 July 2020, T.C. Mu, paratype HSAUP201152, ex-paratype living culture SAUCC201152.

Notes. *Colletotrichum pandanicola* was originally described from the healthy leaves of *Pandanus* sp. (MFLU 18-0003, Pandanaceae) in Thailand (Tibpromma et al. 2018). In the present study, two strains (SAUCC200204 and SAUCC201152) are clustered to the *C. pandanicola* clade in the combined phylogenetic tree (Fig. 1). Morphologically, our strains were similar to *C. pandanicola* by conidia (14.2–17.9 × 4.6–6.0 vs. 9.0–18.0 × 4.0–8.0 μm, mean: 16.1 × 5.4 vs. 13.39 × 5.35 μm). We therefore consider the isolated strains as *C. pandanicola*.

Discussion

In this study, the *Colletotrichum* specimens of diseased leaves and fruits were collected in Mengyin, Shandong Province, China. A temperate monsoon climate and an abundance of fruit trees provide the proper conditions for anthracnose propagation. As a result, 70 reference sequences (including an outgroup taxon: *C. boninense* CBS 123755) were selected based on BLAST searches of NCBI's GenBank nucleotide database and were included in the phylogenetic analyses (Table 2).

Phylogenetic analyses based on seven combined loci (ITS, GAPDH, CHS-1, ACT, TUB2, CAL and GS), as well as morphological characters of the asexual morph obtained in culture, were contributed to knowledge of the diversity of *Colletotrichum* species in Shandong Province. Based on a large set of freshly collected specimens from Shandong province, China, nine strains of *Colletotrichum* species were isolated from two host genera (Table 2). A new species is proposed: *C. mengyinense*. In a previous report, *C. gloeosporioides* has been isolated from *Juglans regia* (Zhu et al. 2014). *Colletotrichum pandanicola* was described from *Pandanus* sp. (Pandanaceae) in Thailand (Tibpromma et al. 2018) and *C. pandanicola* is first reported from *Juglans regia* in China. In this study, we described and illustrated *C. gloeosporioides* and *C. pandanicola* again.

Previously, species identification of *Colletotrichum* was largely referred to the host-specificity and pure culture characteristics, leading to the chaos of names (Weir et al. 2012). On the other hand, based on a polyphasic approach and known morphology, more than one species of *Colletotrichum* can colonize a single host, while one species can be associated with different hosts (Damm et al. 2012). It revealed diversity of *Colletotrichum* species from different hosts. Our study supported this result. For example, *C. pandanicola* (SAUCC200204 and SAUCC201152) and *C. gloeosporioides* (SAUCC200952, SAUCC200954 and SAUCC201001) were collected from *Juglans regia*. In addition, isolates of *C. mengyinense* were obtained from two hosts (*Juglans regia* and *Rosa chinensis*). The morphological descriptions and molecular data for species of *Colletotrichum* represent an important resource and basis for plant pathologists and fungus taxonomists.

Acknowledgements

References

Bhunjun CS, Phukhamsakda C, Jayawardena RS, Jeewon R, Promputtha I, Hyde KD (2021) Investigating species boundaries in *Colletotrichum*. Fungal Diversity 107: 107–127. https://doi.org/10.1007/s13225-021-00471-z

Cai L, Hyde KD, Taylor PWJ, Weir BS, Waller JM, Abang MM, Zhang ZJ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG, McKenzie EHC, Johnston PR (2009) A polyphasic approach for studying *Colletotrichum*. Fungal Diversity 39: 183–204.

Cannon PF, Buddie AG, Bridge PD (2008) The typification of *Colletotrichum gloeosporioides*. Mycotaxon 104: 189–204.

Cannon PF, Damm U, Johnston PR, Weir BS (2012) *Colletotrichum* - current status and future directions. Studies in Mycology 73: 181–213. https://doi.org/10.3114/SIM0014

Chomnunti P, Hongsanan S, Aguirre-Hudson B, Tian Q, Peršoh D, Dhami MK, Alias AS, Xu J, Liu X, Stadler M, Hyde KD (2014) The sooty moulds. Fungal Diversity 66: 1–36. https://doi.org/10.1007/S13225-014-0278-5

Corda ACI (1831) Die Pilze Deutschlands. In: Sturm J (Ed.) Deutschlands Flora in Abbildungen nach der Natur mit Beschreibungen. Sturm, Nürnberg 3(12): 33–64.

Damm U, Cannon PF, Woudenberg JH, Johnston PR, Weir BS, Tan YP, Shivas RG, Crous PW (2012) The *Colletotrichum boninense* species complex. Studies in Mycology 73: 1–36. https://doi.org/10.3114/sim0002

Damm U, Woudenberg JHC, Cannon PF, Crous PW (2009) *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Diversity 39: 45–87.

Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD (2012) The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology13: 414–430. https://doi.org/10.1111/J.1364-3703.2011.00783.X

Gao YH, Sun W, Su YY, Cai L (2013) Three new species of *Phomopsis* in Gutianshan Nature Reserve in China. Mycological Progress 13: 111–121. https://doi.org/10.1007/S11557-013-0898-2

Guo LD, Hyde KD, Liew ECY (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytologist 147: 617–630. https://doi.org/10.1046/J.1469-8137.2000.00716.X

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17(17): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Jayawardena RS, Hyde KD, Jeewon R, Li XH, Liu M, Yan JY (2016) Mycosphere Essay 6: Why is it important to correctly name *Colletotrichum* species? Mycosphere 7: 1076–1092. https://doi.org/10.5943/mycosphere/si/2c/1

Jayawardena RS, Camporesi E, Elgorban AM, Bahkali AH, Yan J, Hyde KD (2017) A new species of *Colletotrichum* from *Sonchus* sp. in Italy. Phytotaxa 314(1): 55–63. https://doi.org/10.11646/phytotaxa.314.1.3

Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20: 1160–1166. https://doi.org/10.1093/bib/bbx108

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/MOLBEV/MSW054

Liu F, Weir BS, Damm U, Crous PW, Wang Y, Liu B, Wang M, Zhang M, Cai L (2015) Unravelling *Colletotrichum* species associated with *Camellia*: employing ApMat and GS loci to resolve species in the *C. gloeosporioides* complex. Persoonia 35: 63–86. https://doi.org/10.3767/003158515X687597

Miller MA, Pfeiffer W, Schwartz T (2012) The CIPRES science gateway: enabling high-impact science for phylogenetics researchers with limited resources. Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment. Bridging from the extreme to the campus and beyond. Association for Computing Machinery 39: 1–8. https://doi.org/10.1145/2335755.2335836

Nylander JAA (2004) MrModeltest v. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Prihastuti H, Cai L, Chen H, Mckenzie EHC, Hyde KD (2009) Characterization of *Colletotrichum* species associated with coffee berries in northern Thailand. Fungal Diversity 39: 89–109.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/BIOINFORMATICS/BTG180

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Stamatakis A (2014) RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Tibpromma S, Hyde KD, Bhat JD, Mortimer PE, Xu J, Promputtha I, Doilom M, Yang JB, Tang AMC, Karunarathna SC (2018) Identification of endophytic fungi from leaves of Pandanaceae based on their morphotypes and DNA sequence data from southern Thailand. MycoKeys: 25–67. https://doi.org/10.3897/mycokeys.33.23670

Tode HJ (1790) Fungi Mecklenburgenses Selecti. Fasc. 1. Nova Fungorum Genera Complectens. https://doi.org/10.5962/bhl.title.148599

Weir BS, Johnston PR, Damm U (2012) The *Colletotrichum gloeosporioides* species complex. Studies in Mycology 73: 115–180. https://doi.org/10.3114/sim0011

Zhu YF, Yin YF, Qu WW, Yang KQ (2014) Occurrence and Spread of the Pathogens on Walnut (*Juglans regia*) in Shandong Province, China. Acta Horticulturae 1050: 347–351. https://doi.org/10.17660/ACTAHORTIC.2014.1050.47